

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
3 January 2002 (03.01.2002)

PCT

(10) International Publication Number
WO 02/00142 A2

(51) International Patent Classification⁷: **A61F 2/44**

(21) International Application Number: **PCT/CA01/00972**

(22) International Filing Date: 29 June 2001 (29.06.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/215,022 29 June 2000 (29.06.2000) US

(71) Applicant (*for all designated States except US*): **MOUNT SINAI HOSPITAL [CA/CA]**; 600 University Avenue, Toronto, Ontario M5G 1X5 (CA).

(72) Inventor; and

(75) Inventor/Applicant (*for US only*): **KANDEL, Rita** [CA/CA]; 430 Heath Street East, Toronto, Ontario M4G 1B5 (CA).

(74) Agent: **BERESKIN & PARR**; 40 King Street West, 40th Floor, Toronto, Ontario M5H 3Y2 (CA).

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:

— without international search report and to be republished upon receipt of that report

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.



WO 02/00142 A2

(54) Title: INTERVERTEBRAL DISC

(57) Abstract: The present invention relates to an engineered biological material comprising or enriched for tissue of intervertebral disc; tissue derived from an engineered biological material; constructs comprising one or more tissues from an engineered biological material; methods for producing the engineered biological materials and constructs; and methods of using the engineered biological materials and constructs.

Title: Intervertebral Disc**FIELD OF THE INVENTION**

The present invention relates to an engineered biological material comprising tissue of intervertebral disc; constructs comprising one or more engineered biological materials; methods for producing the biological materials and constructs; and methods of using the biological materials or constructs.

BACKGROUND OF THE INVENTION

The human spine consists of thirty-three vertebral bodies of which the distal nine are fused to form the sacrum and coccyx (Simon, SR, et al 1994; Bogduk, N., 1997). The 24 vertebrae, with the exception of C1 and C2, are each separated by an intervertebral disc (IVD). The IVD anchors adjacent vertebral bodies and by doing so allows for spinal stabilization, load bearing, and movement. The intervertebral disc is a specialized structure consisting of two interdependent tissues, the annulus fibrosus (AF) and the nucleus pulposus (NP) which merge with the cartilage endplate (Bogduk, N., 1997; Eyre, DR, 1979). The composition of the AF and nucleus pulposus varies with anatomical site in the tissue and the age of the individual (Eyre, DR, 1979; Buckwalter, 1995). The normal function of the disc is dependent on maintenance of the composition, organization, and integrity of the different components (Chiba, 1998). The annulus fibrosus is responsible for withstanding circumferential tensile forces while the nucleus pulposus resists compressive forces during normal activity (Simon, 1994; Bogduk, 1997; Eyre, 1979; Buckwalter, 1995). The disc is relatively avascular as only the outer portion of the annulus contains blood vessels in adults (Buckwalter, 1995). The disc cells rely on diffusion of nutrients from these vessels and from blood vessels in the vertebral body.

The annulus fibrosus surrounds the nucleus pulposus and consists of approximately 10-20 lamellar sheets each composed of collagen fibres oriented parallel to each other and about 65° from the vertical. Although the angle is the same, the direction of the inclination alternates with each sheet such that the fibres in one lamella are 65° to the right, while in the next lamella they are 65° to the left. Every second lamella has the same orientation (Bogduk, N., 1997). This very specific collagen organization allows the disc to rotate and flex. Collagen makes up about 70% of the dry weight of the annulus (Buckwalter, 1995). Type I collagen is the predominate collagen but types II, III, V, VI and type IX collagen are also present in lesser amounts (Bogduk, N., 1997; Buckwalter, 1995; Nerlich, 1998). The average diameter of the collagen fibril is 50-60nm as determined by transmission electron microscopy (Eyre, 1979). The annulus also contains a small amount of proteoglycans and these also have a specific distribution. The proteoglycan content in the tissue is lowest in the periphery of the annulus fibrosus and increases in amount towards the nucleus pulposus (Bogduk, N., 1997). The major proteoglycan is aggrecan (Bogduk, N., 1997; Inerot, 1991; Roberts, 1994; Antoniou, 1996; and Sztrolovics, 1997). Small proteoglycans such as decorin, biglycan, fibromodulin are also present (Gotz, 1997; Sztrolovics, 1999). Elastin and other non-collagenous proteins are detected in the disc (Bogduk, 1997). The cellularity across the annulus varies, as it is more cellular in the outer third (0.7ug DNA/gm dry weight) when compared to the inner two-thirds (0.1ug DNA/gm dry weight) of the annulus (Bayliss, 1998).

The nucleus pulposus (NP) is gelatinous type tissue, which is surrounded by the annulus fibrosus and confined by the cartilaginous endplates of the vertebral bodies (Bogduk, 1997). It consists of

proteoglycans within a loose network of collagen and does not show the same degree of collagen organization in the matrix as the annulus fibrosus (Eyre, 1979; Aguiar, 1999). Proteoglycans comprise approximately up to 65% of the dry weight of the nucleus. Aggrecan is the major proteoglycan present in the nucleus pulposus and about 60% of it is present in a form that does not aggregate. Other proteoglycans, 5 such as decorin, biglycan, and fibromodulin, are also present (Buckwalter, 1995; Gotz, 1997; Sztrolovics, 1999; and Oegema, 1993). The nucleus pulposus contains predominately type II collagen but there are other collagen types present, such as III, VI, IX, and XI (Eyre, 1979; Buckwalter, 1995; Aulisa, 1998). Type I collagen has been detected in small amounts in the nucleus pulposus of humans (Eyre, 1979) and rats (Rufai, 1995). The average diameter of the collagen fibrils is around 30nm as determined by 10 transmission electron microscopy (Eyre, 1979). In childhood, the nucleus contains notochordal cells but these disappear with age. It has been postulated that these cells contribute to the maintenance of the nucleus pulposus and their absence in the adult disc explains the high prevalence of disc degeneration (Aguiar, 1999).

The other component of the disc is the cartilage endplate, a thin layer of articular cartilage that is 15 integrated with the underlying bone of the vertebral body (Bogduk, 1997). As the endplate covers a portion of the vertebral body it confines the nucleus pulposus entirely but only a portion of the annulus fibrosus. The peripheral portion of the annulus fibrosus inserts directly into the bone. The endplate is considered part of the disc as it can easily be separated from the vertebral body (Bogduk, 1997). Like articular cartilage, the endplate consists predominately of water, proteoglycans and collagen (Bogduk, 1997; 20 Antoniou, 1996). The zone of tissue closer to the bone is richer in collagen as compared to the zone closer to the nucleus pulposus, which contains less collagen and more proteoglycans and water (Bogduk, 1997).

Intervertebral disc prolapse is a very common problem and currently there is no optimal treatment for persistent disease. In an autopsy study, 97% of individuals 50 years or older showed disc degeneration (Miller, 1988). It is not known why it is so common, but may be due in part to the relative avascularity of 25 the tissue until there is prolapse (Ozaki, 1999), mechanical factors (Hadjipavlou, 1999), the absence of notochordal cells (Aguiar, 1999) or genetic factors (Kawaguchi, 1999). The back pain that can develop as a result of this disease is often self-limited, but a percentage of affected individuals require surgery (Kraemer, 1994; Borenstein, 1999). Although the surgical intervention may relieve pain faster, this procedure does not restore disc height or its original load bearing capacity. Post-discotomy syndrome, 30 which is characterized by persistent pain and occurs after disc surgery, may be treated by spinal fusion. This is a less than optimal treatment as it is not always successful and results in limited flexibility and degenerative changes in adjacent vertebrae (Javedan, 1999).

IVD replacement, with allografts or prosthetic devices, has been attempted but met with limited success (Leivseth, 1999; Hou, 1991; Enker 1993; Bao, 1996; and Kostiuk, 1997). Alternative treatments 35 such as laser treatment are now being studied (Zeegers, 1999). Intradiscal injection of chymopapain has been used; however, a recent study of 51 patients showed that this treatment had no effect (Choy, 1998). Although not used currently, gene therapy may be another way to treat this disease (Nishida, 1998; Nishida, 1999; and Evans & Robbins, 1999). There is clearly a need to develop novel approaches to the treatment of disc disease.

SUMMARY OF THE INVENTION

The present inventor has produced an engineered biological material comprising or enriched for one or more tissues of intervertebral disc.

In particular, the present inventor has produced an engineered biological material comprising a continuous layer of nucleus pulposus tissue. The tissue formed *in vitro* was characterized and compared to *in vivo* nucleus pulposus tissue and it was found to mimic the organization of nucleus pulposus tissue *in vivo*. In particular, the accumulation of sulfated proteoglycans in the nucleus pulposus tissue continued up to 10 weeks and this was paralleled by an increase in tissue thickness and dry weight. DNA content decreased over time. The amount of DNA and proteoglycans per mg dry weight of the tissue generated in 10 weeks old cultures were substantially the same as for the *in vivo* tissue. There was no significant difference between *in vitro* and *in vivo* tissues. The cells in culture synthesized large proteoglycans which were similar in size to those synthesized by cells in nucleus pulposus tissue explant culture as determined by Sepharose CL-2B column chromatography. The cells also synthesized type II collagen.

Therefore, in one aspect, the invention relates to an engineered biological material comprising a continuous layer of nucleus pulposus tissue. In an embodiment, an engineered biological material is provided comprising in combination a substrate and a continuous layer of nucleus pulposus tissue on the substrate.

In an embodiment, the invention provides an engineered biological material comprising in combination nucleus pulposus tissue and a substrate for the nucleus pulposus tissue, the nucleus pulposus tissue being reconstituted on the substrate *in vitro* from isolated nucleus pulposus cells and being a continuous layer comprising nucleus pulposus cells and an extracellular matrix containing sulfated proteoglycans and type II collagen.

In another aspect, the invention relates to an engineered biological material comprising a continuous layer of annulus fibrosus tissue. In an embodiment the biological material comprises in combination a substrate and a continuous layer of annulus fibrosus tissue on the substrate.

In an embodiment, the invention provides an engineered biological material comprising in combination annulus fibrosus tissue and a substrate for the annulus fibrosus tissue, the annulus fibrosus tissue being reconstituted on the substrate *in vitro* from isolated annulus fibrosus cells and being a continuous layer comprising annulus fibrosus cells and an extracellular matrix.

In another aspect of the invention an engineered biological material is provided comprising a continuous layer of nucleus pulposus tissue surrounded by annulus fibrosus tissue.

The invention also relates to nucleus pulposus tissue and/or annulus fibrosus tissue derived from the engineered biological materials of the invention.

Still further the invention contemplates an intervertebral disc construct comprising nucleus pulposus or annulus fibrosus tissue derived from a biological material of the invention fused to a substrate (e.g. bone substitute). A construct is also provided comprising nucleus pulposus tissue derived from a biological material of the invention fused to a continuous layer of cartilage tissue on a substrate. The nucleus pulposus tissue may be surrounded by a continuous layer of annulus fibrosus tissue.

The invention also relates to a process for producing an engineered biological material of the invention comprising isolating nucleus pulposus cells or annulus fibrosus cells from intervertebral disc;

forming a layer of the nucleus pulposus cells or annulus fibrosus cells on a substrate, and; culturing the nucleus pulposus cells or annulus fibrosus cells in growth media under suitable conditions so that the nucleus pulposus cells or annulus fibrosus cells accumulate extracellular matrix and form nucleus pulposus tissue or annulus fibrosus tissue, respectively.

5 The invention also provides a process for producing an engineered biological material of the invention comprising isolating nucleus pulposus cells and annulus fibrosus cells from intervertebral disc; forming a layer of the nucleus pulposus cells surrounded by the annulus fibrosus cells on a substrate, and; culturing the nucleus pulposus cells and annulus fibrosus cells in growth media under suitable conditions so that the nucleus pulposus cells and annulus fibrosus cells accumulate extracellular matrix and form
10 nucleus pulposus tissue surrounded by annulus fibrosus tissue.

In an embodiment the substrate is selected from the group consisting of bone, an engineered biomaterial preferably an engineered bone substitute, and porous tissue culture inserts.

The nucleus pulposus cells or annulus fibrosus cells in the engineered biological materials or constructs of the invention may be transformed with recombinant vectors containing an exogenous gene
15 encoding a biologically active protein that corrects or compensates for a genetic deficiency, or stimulates cell growth or stimulates extracellular matrix production by cells, or alternatively, encoding a drug. Therefore, the invention also contemplates an engineered biological material or construct of the invention wherein nucleus pulposus cells or annulus fibrosus cells in the biological material or construct are transformed with recombinant vectors containing an exogenous gene encoding a biologically active protein
20 which can correct or compensate for a genetic deficiency or have a stimulatory effect, or encoding a drug.

The invention still further relates to a system for testing a substance or agent that affects nucleus pulposus tissue or annulus fibrosus tissue comprising: generating or culturing a biological material, or construct of the invention in the presence of a substance or agent which is suspected of affecting nucleus pulposus tissue or annulus fibrosus tissue, and determining the biochemical composition and/or
25 physiological organization of tissue generated or cultured, with the biochemical composition and/or physiological organization of the biological material or construct generated or cultured in the absence of the substance or agent.

The invention still further relates to a method of using the biological materials and constructs of the invention to test pharmaceutical preparations for efficacy in the treatment of diseases of intervertebral
30 disc.

Still another aspect of the present invention provides a method of conducting a drug discovery business comprising:

- (a) identifying agents that affect the biochemical composition and/or physiological organization of an engineered biological material of the invention;
- 35 (b) conducting therapeutic profiling of agents identified in step (a), or further analogs thereof, for efficacy and toxicity in animals; and
- (c) formulating a pharmaceutical preparation including one or more agents identified in step (b)
 as having an acceptable therapeutic profile.

In certain embodiments, the subject method can also include a step of establishing a distribution system for distributing the pharmaceutical preparation for sale, and may optionally include establishing a sales group for marketing the pharmaceutical preparation.

Yet another aspect of the invention provides a method of conducting a target discovery business
5 comprising:

- (a) providing one or more engineered biological materials for identifying agents by their ability to affect the biochemical composition and/or physiological organization of the engineered biological material;
- (b) (optionally) conducting therapeutic profiling of agents identified in step (a) for efficacy and
10 toxicity in animals; and
- (c) licensing, to a third party, the rights for further drug development and/or sales for agents identified in step (a), or analogs thereof.

The invention provides methods of using an engineered biological material or tissues obtained therefrom or construct of the present invention as an implant to replace or repair damaged or deficient
15 intervertebral disc, and methods for repairing damaged or degenerated intervertebral discs. Methods of the invention may be used to treat vertebrates suffering from degenerated intervertebral disc conditions, and in particular to treat humans with such conditions.

Therefore, the invention contemplates a method of replacing or repairing damaged or deficient intervertebral discs or portions thereof (preferably nucleus pulposus) of a patient comprising implanting an
20 engineered biological material (or tissue therefrom) or construct of the invention into the site of the damaged or deficient intervertebral disc of the patient. Methods for enhancing healing of an intervertebral disc in a patient are contemplated which comprise inserting a biological material (or tissue therefrom) or construct of the invention into the site of a damaged intervertebral disc.

In an embodiment, the invention provides a method for replacing or repairing a degenerated or
25 damaged nucleus pulposus tissue of an intervertebral disc comprising implanting in the disc space, after the removal of the degenerated or damaged nucleus pulposus tissue, an engineered biological material of the invention comprising a continuous layer of nucleus pulposus tissue or nucleus pulposus tissue obtained therefrom.

In an embodiment, the invention provides a method for replacing or repairing a degenerated or
30 damaged annulus fibrosus tissue of an intervertebral disc comprising implanting in the disc space, after the removal of the degenerated or damaged annulus fibrosus tissue, an engineered biological material of the invention comprising a continuous layer of annulus fibrosus tissue or annulus fibrosus tissue obtained therefrom.

In an aspect of the invention, a method for repairing damaged or degenerated intervertebral discs
35 is provided comprising evacuating tissue from the nucleus pulposus portion of a degenerated intervertebral disc space, preparing a biological material of the invention using nucleus pulposus cells from the evacuated tissue, and implanting the biological material in the evacuated nucleus pulposus space.

In another aspect of the invention, a method for repairing damaged or degenerated intervertebral discs is provided comprising evacuating tissue from the annulus fibrosus portion of a degenerated

intervertebral disc space, preparing a biological material of the invention using annulus fibrosus cells from the evacuated tissue, and implanting the biological material in the evacuated annulus fibrosus space.

The invention also contemplates methods for using the biological materials and constructs of the invention in gene therapy.

- 5 A biological material or construct of the invention can be used as an *in vitro* model for investigating the metabolism and degeneration of nucleus pulposus or annulus fibrosus cells and tissues.

These and other aspects of the present invention will become evident upon reference to the following detailed description and attached drawings.

DESCRIPTION OF THE DRAWINGS

- 10 The invention will be better understood with reference to the drawings in which:

Figure 1A is a photomicrograph of toluidine blue-stained section of 10 wk old formalin-fixed, paraffin-embedded nucleus pulposus cell cultures showing the formation of nucleus pulposus tissue. The filter is still present.

- 15 Figure 1B is a photomicrograph of *in vitro* formed 8 week old nucleus pulposus tissue (toluidine blue, magnification x 600).

Figure 2 shows the determination of culture tissue thickness over time. The cultures were harvested, paraffin-embedded and histological sections were used to measure the nucleus pulposus tissue thickness. The results are expressed as mean \pm SEM of three separate experiments and each time point was done in triplicate.

- 20 Figure 3 shows the determination of culture tissue dry weight over time. The cultures were harvested, lyophilized and weighed. The results are expressed as mean \pm SEM of three separate experiments and each time point was done in triplicate.

- 25 Figure 4 shows the measurement of DNA content of the nucleus pulposus tissue in culture over time. The results are expressed as mean \pm SEM of three separate experiments and each time point was done in triplicate.

Figure 5 shows the proteoglycan content of the nucleus pulposus tissue in culture over time which was determined by measuring glycosaminoglycan content. The results are expressed as mean \pm SEM of three separate experiments and each time point was done in triplicate.

- 30 Figure 6 shows proteoglycan elution profiles of newly synthesized proteoglycans extracted from 10 week old nucleus pulposus cultures (●-●) and of proteoglycans extracted from nucleus pulposus *ex-vivo* cultures (○-○). The size (K_{av}) of newly synthesized proteoglycan monomers was determined by gel chromatography (Sephadex G-200) under dissociating conditions.

The present invention relates to an engineered biological material comprising a continuous layer of nucleus pulposus tissue. The tissue is characterized by nucleus pulposus cells that synthesize large sulfated proteoglycans and type II collagen characteristic of nucleus pulposus cells *in vivo*. The tissue in the biological material is also characterized by becoming less cellular with age. The nucleus pulposus 5 tissue is further characterized by having a three dimensional organization that is characteristic of nucleus pulposus tissue *in vivo*.

In an embodiment, the engineered biological material comprises a continuous layer of nucleus pulposus tissue with a thickness of between about 100 to 500 μm after histological processing.

The present invention also relates to an engineered biological material comprising a substrate and 10 a continuous layer of nucleus pulposus tissue on the substrate.

The invention also relates to an engineered biological material comprising a substrate and a continuous layer of annulus fibrosus tissue on the substrate. The annulus fibrosus cells are characterized by being capable of synthesizing types I and II collagen and proteoglycans similar in size to those synthesized by annulus fibrosus cells *in vivo*. The annulus fibrosus tissue is further characterized by having a three 15 dimensional organization that is characteristic of annulus fibrosus tissue *in vivo*.

The invention also relates to a method for producing an engineered biological material of the invention comprising isolating nucleus pulposus cells or annulus fibrosus cells of intervertebral disc; forming a layer of the cells on a substrate; culturing the cells in growth media under suitable conditions so that the cells accumulate intracellular matrix and form a continuous layer of nucleus pulposus or annulus 20 fibrosus tissue.

The cells used in the method of the invention may be isolated from intervertebral discs (lumbar discs, thoracic discs, or cervical discs) from animals, preferably humans, bovines, ovines, rabbits, most preferably humans. The tissue may be isolated from adult or fetal tissue. In one embodiment of the invention, the cells are isolated from intervertebral disc of the lumbar spine of sheep. Intervertebral disc 25 tissue may be extracted from a patient being treated, or alternatively from a donor, using known surgical techniques.

The nucleus pulposus or annulus fibrosus cells may be isolated from intervertebral disc tissue by sequential enzyme digestion techniques, such as those described in Boyle et al, Osteoarthritis and Cartilage 3, 117-125, 1995. For example, the cells may be treated with 0.5% protease followed by 0.1% bacterial 30 collagenase.

In accordance with the method of the invention a continuous layer of cells is placed on a substrate. Suitable substrates include bone, engineered biomaterials, and porous tissue culture inserts, for example filter inserts.

The substrate is optionally coated with an attachment factor. Attachment factors are known in the 35 art, see for example, Streuli and Bissell, J. Cell. Biol. 110:1405, 1990 and Buck and Horwitz, Ann. Rev. Cell Biol. 3:179, 1987. Examples of attachment factors include type I collagen, type II collagen, type IV collagen, a synthetic peptide of a segment of collagen, (e.g. a fifteen amino acid sequence 766GTPGPQGIAGQRGVV780 which is present in the $\alpha 1$ chain of collagen) (Bhatnagar and Qian, 38th Annual Meeting of the Orthopedic Research Society 17:106, 1992), fibronectin, gelatin, laminin, polylysine, vitronectin, cytотactин, entactin, tenascin, thrombospondin, uvomorulin, biglycan,

chondroitin sulfate, decorin, dermatan sulfate, and heparin. A preferred attachment factor that may be used in the method of the invention is collagen, most preferably type II collagen. When the substrate is coated it may be air dried and sterilized.

5 In a preferred embodiment of the invention the substrate is a tissue culture insert known as Millicell CM®, (Millipore Corp., Bedford, MA, U.S.A.), pore size 0.4µm, coated with an attachment factor, preferably type II collagen (Sigma Chemical Co., St. Louis, MO, U.S.A.).

The substrate may be a bone substitute, in particular an engineered bone substitute such as coral derivatives (Interpore International Inc., CA), deproteinized bovine bone (Bio-oss®, Geistlich Biomaterials, Switzerland), or a porous biodegradable biomaterial which is formed from sintered calcium 10 polyphosphate (CPP) as described in U.S. Serial No. 6,077,989 to Kandel et al. and PCT Application No. PCT/CA97/00331 (WO97/45147 published December 4, 1997). A sintered porous CPP can be formed with a preferred pore size and percent porosity through selection of sintering parameters (time, temperature, starting particle size). Thus, structures can be formed that more closely mimic the structure of the bone into which a composite construct of the invention may be placed. Additionally, degradation rates 15 can be controlled to some extent through control of percent porosity and pore size (i.e. total free surface area). In an embodiment, the substrate is an appropriately-sized porous disc (e.g. 4mm diameter and 3mm thick) that may be encased with an inert tubing to help cell retention by preventing overflow.

Cartilage can be formed on, and anchored to, an engineered bone substitute, preferably a porous CPP substrate. Articular chondrocytes may be cultured on a porous CPP disc to form a continuous layer of 20 cartilagenous tissue using the method described in U.S. Patent No. 5,326,357.

Nucleus pulposus or annulus fibrosus cells may be seeded on a selected substrate at a cell density of about to 1×10^5 to 8×10^6 cells/cm², preferably $2-4 \times 10^6$ cells/cm², more preferably $3-3.5 \times 10^6$ cells/cm², most preferably 3.3×10^6 cells/cm². The cells seeded on a coated or uncoated substrate are grown in suitable culture conditions. Examples of suitable culture media are known in the art, such as Ham's F12 25 and/or Dulbecco's modified Eagle's medium (DMEM). Preferably DMEM is used. The culture medium may contain serum, for example, heat inactivated fetal bovine serum in a concentration range of about 2-20%, preferably 10-20%, and may further contain growth factors, and optionally ascorbic acid. The culture media is applied above and below the substrate. The cells may be cultured at 37°C in a humidified atmosphere supplemented with CO₂.

30 In a preferred embodiment of the invention, the isolated cells are grown in DMEM containing 10% fetal bovine serum for about 5 days, the medium is then changed to DMEM containing 20% fetal bovine serum, and ascorbic acid (100 µg/ml, final concentration) at about 7 days.

The cells are cultured for an additional 5 weeks to obtain the engineered biological material described herein. The cells may be cultured for less than 5 weeks, or greater than 5 weeks, to obtain a 35 product which may be suitable for some uses such as transplantation or gene therapy.

Mechanical force(s) may be administered during *in vitro* formation of the engineered biological material in order to enhance the development of tissues that are highly suited for implantation and physiological weight bearing. Torsion, compression, and/or shear forces may be applied during tissue formation. Forces, together or alone, may be applied, consecutively, simultaneously, or cyclically. The 40 mechanical forces may be applied through the use of a mechanical stimulation system that allows for

loading cell cultures under sterile conditions. For example, the Mach-1™ system (Biosyntech, Montreal) is capable of supplying simultaneous compressive and linear shear forces, and can include the application of torsional shear forces. For each type of force application, a skilled artisan can determine the optimal conditions to induce tissue growth and organization (i.e. force amplitude, frequency and duration of stimulation).

In an embodiment of the invention, either sinusoidal compressive or torsional forces are applied to the developing tissue. Compressive forces may be applied at about day 3, in a range of unconstrained loading between 0.1 to 10 N (approximately corresponding to compressive stresses of 0.01 to 1 MPa), through a compliant, biocompatible, autoclavable elastomer (e.g. medical grade silicone or polyurethane) placed on the actuator to avoid direct contact with the cells. The duration of loading may range from 100 to 1200 cycles/day and may be applied at a frequency of 1.0 Hz. (1 Hz approximates normal gait frequency of disc loading). Minimal numbers of loading cycles may be preferred to stimulate organization of IVD tissues. For example, 20 sec. of 1MPa of hydrostatic pressure may be sufficient to stimulate proteoglycan synthesis by inner annulus cells.

Torsional shear force application may consist of a compressive preload followed by varying degrees of cyclic torsional shear. Angular deformation amplitudes ranging from 0.005 rad to 0.05 rad at a frequency of 1 rad/sec, may be used (approximately corresponding to a maximal torque of 0.5N.mm). Cyclic compressive and torsional shear forces may be simultaneously applied.

The invention also contemplates an intervertebral disc construct. The construct may comprise one or both of annulus fibrosus and nucleus pulposus tissue, with cartilagenous tissue and/or a substrate (e.g. bone substitute). In one embodiment, the construct comprises an engineered bone substitute with cartilagenous tissue formed thereon, and nucleus pulposus tissue derived from an engineered biological material of the invention fused to the bone substitute-cartilagenous tissue. This construct may be prepared by culturing articular chondrocytes on porous CPP discs for about 3 weeks using the methods described in U.S. Patent No. 5,326,357. Simultaneously, nucleus pulposus cells may be grown on a substrate, preferably a filter insert, more preferably a Millipore CM® filter as described herein. At about 3 weeks, a piece of nucleus pulposus tissue formed *in vitro* may be punched out from the substrate (e.g. Millipore CM® filter), and placed on the CPP-cartilagenous tissue construct. The tissue components may be held together using fibrin glue, or other suitable adhesive, and maintained in culture for about 3 weeks. The composite may be harvested to form the construct.

In another embodiment of the invention, the construct resembles a natural disc. Thus, a substrate (e.g. bone substitute) may be made with a central depression, and articular cartilage tissue may be cultured in the depression. Articular cartilage tissue may be cultured in the depression using the methods described in U.S. Patent No. 5,326,357. Annulus fibrosus tissue derived from an engineered biological material of the invention (or other source) may be grown on the cartilagenous tissue formed on the substrate. After fusion of the annulus fibrosus and cartilagenous tissues, a plug of annulus fibrosus tissue may be removed from the centre of the annulus fibrosus tissue and replaced with nucleus pulposus tissue derived from an engineered biomaterial of the invention. The resulting composite comprising annulus fibrosus, nucleus pulposus, cartilage endplate, and substrate is grown in culture to produce a construct comprising fused annulus fibrosus tissue, nucleus pulposus tissue, and cartilage tissue, with a substrate.

In a specific embodiment of the invention a calcium polyphosphate porous cylinder may be generated that has a central depression on its surface. Articular chondrocytes may be isolated (e.g. from sheep knee joint) (Boyle et al, Osteoarthritis and Cartilage 3, 117-125, 1995) and plated in the depression. The cells may be grown under the tissue culture conditions described in U.S. Patent No. 5,326,357 for 5 about 3 weeks during which time they will form cartilagenous tissue. Annulus fibrosus and nucleus pulposus cells may be isolated from sheep lumbar spine. The nucleus pulposus cells may be plated on a substrate, preferably filter inserts (e.g. Millicell CM^R, Millipore Corp) and grown as described herein for about 4 weeks. At about 4 weeks, the nucleus pulposus tissue formed *in vitro* has sufficient strength to be handled. The annulus fibrosus cells are plated on top of the cartilagenous tissue which has formed on the 10 CPP porous cylinder. This composite may be grown in culture for about 4 weeks under the optimal loading conditions as described herein. At about 4 weeks, a plug of annulus fibrosus tissue (e.g. 1-3mm, preferably 2 mm diameter) is punched out from the centre and a plug of nucleus pulposus tissue (obtained from the nucleus pulposus tissue culture) is placed in the resulting defect. This composite consisting of annulus fibrosus, nucleus pulposus, cartilage endplate and CPP may be grown in culture for an 15 additional 4 to 6 weeks as described herein.

In an embodiment, nucleus pulposus cells are plated in the center of a substrate (e.g. disc) with annulus cells surrounding them, and grown in culture together in the presence or absence of mechanical stimulation.

The engineered biological material and constructs of the present invention can be used as model 20 systems for *in vitro* studies of intervertebral disc (or components thereof i.e. annulus fibrosus and nucleus pulposus tissue) function and development.

In accordance with one embodiment of the invention, an engineered biological material, may be used to test substances which affect intervertebral disc or components thereof (e.g. nucleus pulposus or annulus fibrosus tissue). A system for testing a substance that affects intervertebral disc in accordance 25 with the invention comprises generating or culturing an engineered biological material or construct of the invention in the presence of a substance which is suspected of affecting intervertebral disc or components thereof, and determining the biochemical composition and/or physiological organization of tissue generated or cultured, and comparing with the biochemical composition and/or physiological organization of the engineered biological material in the absence of the substance.

The substance may be added to the culture or the cells in the engineered biological materials 30 (nucleus pulposus or annulus fibrosus cells) may be genetically engineered to express the substance i.e. the cells may serve as an endogenous source of the substance. Cells may be engineered by viral or retroviral-mediated gene transfer using methods known in the art to produce a specific substance. The engineered cells are constructed and maintained such that they release the substance into the medium for 35 the desired period of time for the culture.

The system may be used to analyze the effects of substance(s) on different stages of intervertebral disc development. Effects on cells at very early, intermediate, and late stages of development may be evaluated by assessing the biochemical composition and/or physiological organization of the tissue generated in the cultures at various times such as 2, 4, 6 and 8 weeks.

The biochemical composition and/or physiological organization of the tissue generated in the cultures may be assessed using the methods described herein. (See, for example, the methods described in Example 1)

In a preferred embodiment of the invention, the biological materials of the present invention may 5 be used in the testing of pharmaceutical preparations useful in the treatment of diseases of intervertebral disc.

The biological materials of the invention may also be implanted into patients to replace or repair 10 damaged or deficient intervertebral disc. In particular, the biological materials of the invention may be implanted into individuals with idiopathic scoliosis, herniated disc, degenerative disc disease, recurrent disc herniation, or spinal stenosis.

It is also contemplated that the biological materials of the present invention can be used to enhance healing of damaged or deficient intervertebral discs when inserted into the site of the disc.

The invention also contemplates using the biological materials of the invention in gene therapy. Therefore, recombinant vectors containing an exogenous gene encoding a biologically active protein that is 15 selected to modify the genotype and/or phenotype of a cell to be infected may be introduced into cells in the biological materials of the invention. An exogenous gene coding for a biologically active protein which corrects or compensates for a genetic deficiency or a drug may be introduced into cells in the biological materials. For example, IGF-I could be introduced into the cells so that the cells secrete this protein and stimulate production of proteoglycans resulting in disc regeneration. The expression of the 20 exogenous gene may be quantitated by measuring the expression levels of a selectable marker encoded by a selection gene contained in the recombinant vector.

The following non-limiting examples are illustrative of the present invention:

EXAMPLE 1

The following materials and methods were utilized in the investigations outlined in the 25 examples:

Materials and Methods

Cell cultures

The lumber spines from up to 9 month-old sheep were removed. The muscle tissue was cleared from the ventral portion of the spine and then the ligamentous tissue surrounding the disc was carefully excised 30 aseptically and discarded. The annulus fibrosus (AF) and nucleus pulposus (NP) were identified and the nucleus pulposus was dissected out with blunt forceps and placed in Ham's F12. The accuracy of the dissection was determined by submitting random tissue fragments for paraffin embedding and histological assessment at each harvesting.

The tissue underwent sequential enzyme digestion consisting of 0.5% protease (Sigma Chemical 35 Co., St. Louis, MO, USA) for 1 hr at 37°C, followed by 0.1% collagenase (Boehringer Mannheim GmbH, Indianapolis IN, USA) overnight at 37°C. The cell suspension was then filtered through a sterile mesh and plated at a cell density of $3.3 \times 10^6/\text{cm}^2$ on filter inserts (Millicell CMR® Millipore Corp., Bedford, MA) precoated with type II collagen (Sigma Chemical Co., St. Louis, MO, USA) as described previously (Kandel 1997, Yu 1997). The cells were grown in Dulbecco's Modified Eagles medium (DMEM) 40 supplemented with 10% fetal bovine serum (FBS). After 5 days the FBS was increased to 20% and

ascorbic acid (100 µg/ml, final concentration) was added to the medium at day 7. The medium was changed every 2-3 days and fresh ascorbic acid was added each time. The cultures were harvested at 2, 4, 6, 8, and 10 weeks for histologic assessment and biochemical analysis.

Histology

5 The nucleus pulposus cell cultures were harvested at selected intervals and fixed in 10% formalin. They were paraffin-embedded and 5 µm sections were cut and stained with either hematoxylin and eosin or toluidine blue. Toluidine blue stains sulphated proteoglycans.

10 The culture tissue thickness was measured morphometrically using light microscopy and a digitized board connected to an IBM computer equipped with the Bioquant Image Analysis program. Ten separate points in each section were examined and three sections per culture were quantified. Tissue thickness was calculated by determining the mean value of three separate experiments.

Determination of dry weight of culture tissue

15 The cultures, harvested at various times, and representative fragments of the *in vivo* nucleus pulposus were lyophilized overnight. The lyophilized tissues were weighed on an electrical balance (Mettler Instruments, AG, Greifensee-Zurich, Switzerland).

DNA Quantification

20 The cultures at different time points and representative fragments of the *in vivo* nucleus pulposus tissue were digested with papain [40 µg/ml in buffer consisting of 20 mM ammonium acetate, 1mM ethylenediaminetetraacetic acid (EDTA) and 2mM dithiotreitol] for 48 hours at 65°C. The DNA content was measured using Hoescht 33258 dye (Polysciences, Inc., Warrington, PA) and fluorometry (emission wavelength 365 nm and excitation wavelength 458nm) as described by Kim et al. (1988). Calf thymus DNA (Sigma Aldrich Co., St. Louis, MO, USA) was used to generate the standard curve.

Quantification of Proteoglycans and Collagen

25 Proteoglycan content in the *in vitro* formed tissue and the *in vivo* tissue was determined by measuring the amount of glycosaminoglycans in the papain digest using the dimethylmethyle blue dye binding assay (Polysciences Inc., Warrington, PA) and spectrophotometry as described by Farndale et al. (1986) and modified by Goldberg et al (1990). Chondroitin sulphate (Sigma Aldrich Co., St. Louis, MO, USA) was used to generate the standard curve.

30 The collagen content was determined by measuring the amount of hydroxyproline in the papain digests of the cultures. An aliquot was hydrolyzed overnight at 110° using 12N hydrochloric acid. The hydroxyproline was measured by high pressure liquid chromatography using a C18 reverse column and a Waters PicoTag amino acid analysis system. The amount of collagen was calculated from the hydroxyproline content which comprises about 10% of the weight of collagen (Berg, RA, 1982, Determination of 3 and 4 hydroxyproline. In Methods of Enzymology pp. 393-94, Ed. by L.W. Cunningham and D.W. Frederiksen, New York, Academic Press, 1982).

Analysis of newly synthesized proteoglycans

35 To analyze proteoglycan biosynthesis, 8 week old cultures were incubated with [³⁵S]-sulphate (4 µCi/per well) for 24 hours prior to harvesting. Matrix proteoglycans were extracted with 4M guanidine hydrochloride in 50 mM sodium acetate, pH 5.8 containing protease inhibitors (0.1M 6-amino-hexanoic acid, 50mM benzamidine HCl, 10mM EDTA and 5mM N-ethylmaleimide) for 24 hours at 4°C. The

proteoglycans were precipitated by the addition of three volumes of ice cold ethanol. After 24 hours at 4°C the precipitate was collected, washed with 70% ethanol, and resuspended in 4 M guanidium HCl with protease inhibitors. Proteoglycan synthesis was determined by quantitating [³⁵S]-sulphate incorporation by a β-scintillation counter. The proteoglycan size was determined by Sepharose CL-2B column chromatography (1 x 100 cm) under dissociative conditions, as described previously (Boyle 1995). Fractions (2 ml) were collected using a flow rate of 6 ml/hour at 4°C. The elution profile was analyzed for its partition coefficient [Kav=(Ve-Vo)/Vt-Vo), where Vo=void volume, Vt=total volume, Ve=elution volume]. Vt was determined using [³⁵S]-sulphate and Vo was determined using dextran blue 2000.

Sheep *ex vivo* nucleus pulposus explant cultures were established as controls. Immediately after being placed in culture the cultures were labelled for 24 hours with [³⁵S]-sulphate and proteoglycans were extracted identically as described for the filter cultures.

Analysis of collagens

The 8 week old cultures were harvested and digested with pepsin (200 µg/ml in 0.1 N acetic acid; Sigma Aldrich Co., St. Louis, MO, USA) for 72 hours at 4°C. The pepsin extracts were separated on a 5 % sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and either silver stained (Silver Stain Plus Kit, Bio-Rad, Hercules, CA) or transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH, USA) for Western blot analysis. The presence of type II collagen was determined by Western blot using antibody reactive with type II collagen (4 µg/ml. monoclonal clone 6B3; NeoMarkers, Union City, CA, USA). Immunoreactive bands were visualized by a chemiluminescence using peroxidase-labelled secondary antibodies (Western blotting kit, Boehringer Mannheim GmbH, Indianapolis, IN, USA).

Statistical Analysis

Student's t-test was used to analyse the data and significance assigned at a p value <0.05.

Results

Histologic appearance of cultures

The nucleus pulposus cultures were examined histologically at 2, 4, 6, 8, and 10 weeks after initiation of the cultures to assess matrix accumulation and tissue organization. By 2 weeks in cultures, the nucleus pulposus cells accumulated extracellular matrix to form a continuous layer of tissue which contained sulfated proteoglycans as demonstrated by toluidine blue staining. Histologically, the extracellular matrix tissue appeared more abundant over time in culture (Figure 1A). The cells were in lacunae similar to the *in vivo* nucleus pulposus tissue (Figures 1B). The tissue thickness was measured in the histological sections, and it increased in thickness up to 10 weeks in culture. The nucleus pulposus tissue which had been dehydrated and paraffin embedded, attained a thickness of 308.8 ± 29 µm (mean ± SEM) at 10 wk. (Figure 2).

Tissue dry weight

The dry weight of the *in vitro* nucleus pulposus tissue increased over time up to 10 weeks. The dry weight of the tissue, which was 0.87 ± 0.1 mg per culture (mean ± SEM) at 2 weeks, weighed 2.16 ± 0.2 mg per culture (mean ± SEM) by 10 weeks (Figure 3).

DNA content of the nucleus pulposus tissue

The DNA content of the nucleus pulposus tissue formed in culture was stable during the first 4 weeks in culture but then decreased over the following 6 weeks of culture. By 10 weeks the tissue

cellularity had plateaued. (Figure 4). The tissue formed *in vitro* at 10 weeks contained 1.25 ± 0.02 µg DNA/mg dry weight (mean \pm SEM), whereas the *in vivo* nucleus pulposus contained 1.04 ± 0.08 µg DNA/mg dry weight (mean \pm SEM). There was no significant difference between the *in vitro* formed tissue and the *in vivo* tissue.

5 Quantification of proteoglycan and collagen content

Proteoglycans and collagen are the major macromolecules of the nucleus pulposus tissue. The proteoglycan and collagen contents in the extracellular matrix of the nucleus pulposus tissue in culture were quantified in order to examine matrix accumulation. The proteoglycan content, as determined by measuring glycosaminoglycan content, increased up to 10 weeks (Figure 5). The tissue formed *in vitro* at 10 weeks contained 301.6 ± 27.7 µg GAG/mg dry weight (mean \pm SEM), and 411 ± 65 µg collagen/mg dry weight (mean \pm SEM), whereas the *in vivo* nucleus pulposus contained 320.6 ± 21.2 µg GAG/mg dry weight (mean \pm SEM) and 399 ± 44 µg collagen/mg dry weight (mean \pm SEM) (Table 1). There was no significant difference between the *in vitro* formed tissue and the *in vivo* tissue.

Analysis of the proteoglycans and collagens

15 As large proteoglycans and type II collagen are the main macromolecules present in the matrix of nucleus pulposus tissue, the proteoglycans and collagens present in the nucleus pulposus tissue formed *in vitro* were analyzed to determine whether the phenotype of these cells was retained under these culture conditions. To determine the size of the proteoglycans retained in the matrix, the [35 S]-sulphate labeled proteoglycans were guanidinium extracted from 8 week old culture. As shown in Figure 6, analysis by 20 column chromatography under dissociative conditions demonstrated that the proteoglycan monomers have a large hydrodynamic size ($K_{av} = 0.26 \pm 0.03$, mean \pm SD), which was similar in size to those synthesized by cells in the *ex vivo* tissue cultures ($K_{av} = 0.22 \pm 0.02$, mean \pm SD, $p > 0.05$). Nucleus pulposus cells *in vitro* and *in vivo* were also synthesizing a smaller population of proteoglycan with a K_{av} around 0.7.

25 Pepsin extracts of 10 week old tissue formed *in vitro* were analyzed by SDS-PAGE and autoradiography. A band similar in size to the $\alpha 1(II)$ chain of type II collagen was seen. Western blot analysis of these extracts confirmed the presence of type II collagen (Figure 7).

Discussion

30 A cell culture system is described herein in which nucleus pulposus cells isolated from sheep lumbar spines and grown on Millipore CM[®] filter inserts accumulate extracellular matrix and form a continuous layer of nucleus pulposus tissue. The nucleus pulposus cells in these cultures maintained their phenotype as they synthesized large sulfated PGs and type II collagen which are characteristic of nucleus pulposus cells. Cells isolated from rabbit nucleus pulposus also generated tissue in culture under similar conditions.

The composition of the *in vitro* formed tissue was similar to the *in vivo* nucleus pulposus tissue. 35 The cellularity and proteoglycan content at 10 weeks was comparable to the *in vivo* tissue. The predominate proteoglycan in the nucleus pulposus is aggrecan (Melrose 1994, Oegema 1979), and the cells in filter culture synthesized large proteoglycans in keeping with this type of proteoglycan. The size of proteoglycans synthesized by the nucleus pulposus cells *in vitro* was similar to that synthesized by the nucleus pulposus cells in *ex-vivo* culture as determined by column chromatography. As well a smaller 40 amount of proteoglycans with a K_{av} of approximately 0.7 were also detected. Proteoglycans of this size

may represent degradation products or the presence of the small proteoglycans, such as decorin, fibromodulin, which have been shown to be present in the nucleus pulposus (Melrose 1994; Inkinen 1998; Jahnke 1988; Sztrolovics 1999; Gotz, 1997).

The cellularity of the *in vitro* tissue decreased between 4 and 10 weeks of culture and in doing so 5 more closely approximates the cellularity of the *in vivo* tissue. The nucleus pulposus *in vivo* has been shown to become less cellular with age (Trout 1982; Buckwalter 1995). Other studies have shown that DNA content increases with time when nucleus pulposus cells were cultured in either monolayer or alginate gel. In these culture systems the cells do not synthesize sufficient matrix to form a continuous layer of tissue, so it is possible that the nucleus pulposus cells are in a different microenvironment than 10 those present in the *in vivo* tissue (Chiba 1997, Sato 1999, Ichimura 1991).

This new culture system has several advantages over other culture systems in that the nucleus pulposus cells do not dedifferentiate, as indicated by the maintenance of their phenotype during the time period studied, and form a continuous layer of tissue which is amenable to histological and biochemical assessment. In addition the nucleus pulposus cells under these culture conditions maintain their phenotype. 15 Other methods to culture nucleus pulposus cells have been described and include growing cells in monolayer (Ichimura 1991) or encapsulated within alginate (Chelberg 1995; Chiba 1997, Sato 1999 Maldonado 1992). However, these do not result in tissue formation and so do not mimic the organization of the nucleus pulposus tissue i.e. they do not accumulate sufficient extracellular matrix to form a continuous layer of tissue and so do not form a three dimensional structure. For example, nucleus pulposus 20 cells grown in monolayer or suspension culture do not accumulate sufficient extracellular matrix to form a continuous layer of tissue (Ichimura 1991, Osada 1996). Nucleus pulposus cells grown in alginate or agarose beads remain spherical and accumulate mainly type II collagen and large proteoglycans but still do not form tissue (Chiba 1997, 1998, Maldonado 1992, Chelberg 1995). Furthermore, it has been shown that molecules of up to 200,000 molecular weight are able to diffuse out of alginate and this may influence 25 what is retained within the matrix of these cultures (Kupchik 1983). Kusior LJ et al (1999) has reported that nucleus pulposus cells embedded in biocompatible polymers, such as polyglycolic acid and calcium alginate, will form tissue. However, the cell seeded scaffolds had to be implanted in nude mice for tissue formation to occur. Although organ culture of the intervertebral disc has been used to study metabolism of the whole tissue it can not be maintained at a comparable metabolism level to the *in vivo* tissue. (Urban 30 1981, Thompson 1991). Recently an alternative approach was described by Chiba et al. (1998) in which an intact disc was surrounded with alginate, however this method did not entirely inhibit the matrix loss that occurs during the first few days of culture.

In conclusion, these studies demonstrate that the nucleus pulposus cells grown on filters generate tissue similar to the *in vivo* tissue for the features examined.

35 **EXAMPLE 2**

Formation of disc construct

The possible formation of a composite of nucleus pulposus tissue fused to cartilage which is anchored to the porous CPP was investigated. Articular chondrocytes were plated on the porous CPP discs and allowed to grow for 3 weeks. Simultaneously nucleus pulposus cells were grown on Millipore CM^R 40 filters as described above. At 3 weeks, a piece of nucleus pulposus tissue formed *in vitro* was punched out

from the Millipore CM^R filter and placed on the CPP-cartilagenous tissue construct that had been prepared. The tissue components were held together using fibrin glue and maintained in culture for 3 weeks. The composite was harvested and light microscopical examination of the processed constructs showed that substantial fusion of nucleus pulposus tissue with the underlying cartilagenous tissue occurred.

5 **EXAMPLE 3**

Generation of an intervertebral disc- CPP biomaterial construct:

One half of a spinal unit consists of an intervertebral disc fused to a cartilage endplate integrated with subchondral bone. To more closely mimic a natural disc, CPP will be generated with a central depression on its surface, the diameter of which will depend on the diameter of the CPP cylinder and with 10 a depth of 0.5 mm. Articular chondrocytes can be isolated from sheep knee joint as described previously (Boyle et al, 1995) and plated in this depression. The cells will be grown under standard tissue culture conditions for 3 weeks during which time they will form cartilagenous tissue. Annulus fibrosus and nucleus pulposus cells will be isolated from sheep lumbar spine. The nucleus pulposus cells will be plated on filter inserts (Millicell CM^R, Millipore Corp) and grown as described above for 4 wks (see Example 1). 15 At 4 weeks the nucleus pulposus tissue formed *in vitro* has sufficient strength to be handled. The annulus fibrosus cells will be plated on top of the cartilagenous tissue which has formed on the CPP porous cylinder. This composite will be grown in culture for 4 weeks under the optimal loading conditions described herein. At 4 weeks a 2mm diameter plug of annulus fibrosus tissue will be punched out from the centre and a plug of nucleus pulposus tissue (obtained from the nucleus pulposus tissue culture) will be 20 placed in the resulting defect. In preliminary studies, no dead cells were seen at the edge of the punched out nucleus pulposus tissue suggesting that this manipulation does not damage the tissue. This composite consisting of annulus fibrosus, nucleus pulposus, cartilage endplate and CPP will then be grown in culture for an additional 4 to 6 wks under loading as defined above.

25 Having illustrated and described the principles of the invention in a preferred embodiment, it should be appreciated to those skilled in the art that the invention can be modified in arrangement and detail without departure from such principles. All modifications coming within the scope of the following claims are claimed.

30 All publications, patents and patent applications referred to herein are incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety.

Table 1

5

Comparison of 10 week old *in vitro* generated nucleus pulposus tissue with *in vivo* nucleus pulposus tissue

	<i>in vitro</i>	<i>in vivo</i> (μ g/mg dry weight)
10	DNA	1.25 ± 0.02
	GAG	301.6 ± 27.7
15	COLLAGEN	411 ± 65
		399 ± 44

The dry weight, and DNA, glycosaminoglycans (GAG) and collagen (hydroxyproline) contents were determined as described in Example 1. The analyses were performed on three different samples from each of three separate experiments. The data are expressed as mean \pm SEM of the three experiments.

References:

- Simon SR, et al. In: Orthopedic Basic Science Ed. SR Simon. Am Academy of Orthopedic Surgeons, USA, 1994, 558-568
- Bogduk N. The inter-body joints and the intervertebral discs. In: Bogduk N, Ed, Clinical Anatomy of the lumbar spine and sacrum. Churchill Livingstone, New York, 1997, pp:13-31
- Eyre DR. Biochemistry of the Intervertebral Disc. Int Rev Conn Tiss Res 8:227-291, 1979
- Buckwalter JA. Aging and Degeneration of the Human Intervertebral Disc. Spine 20(11):1307-14, 1995
- Chiba K, Andersson GBJ. Masuda K., Momohara S. Williams JM, Thonar E J-M A. A New Culture System to Study the Metabolism of the Intervertebral Disc In Vitro Spine 23(17):1821-1828, 1998
- Nerlich A, Boos N, Wiest I, Aebi M. Immunolocalization of major interstitial collagen types in human lumbar intervertebral discs of various ages. Virchows Arch 432:67-76, 1998
- Inerot S, Axelsson I. Structure and Composition of Proteoglycans from Human Annulus Fibrosus. Conn Tiss Res 26:47-63, 1991
- Roberts S, Caterson B, Evans H, Eisenstein, SM. Proteoglycan components of the intervertebral disc and cartilage endplate: an immunolocalization study of animal and human tissues. Histochem J 26:402-11, 1994
- Antoniou J, Steffen T, Nelson F, Winterbottom N, Hollander AP, Poole R A, Aebi M. The Human Lumbar Intervertebral Disc. Evidence for Changes in the Biosynthesis and Denaturation of the Extracellular Matrix with Growth Maturation, Aging and Degeneration. J Clin Invest 98(4): 996-1103, 1996
- Sztralovics R, Alini M, Roughley PJ, Mort, JS. Aggrecan degradation in human intervertebral disc and articular cartilage. Biochem J. 15:326(Pt.1):235-41, 1997
- Gotz W, Barnert S, Bertagnol, R, Miosge N, Kresse H, Herken, R. Immunohistochemical localization of the small proteoglycans decorin and biglycan in human intervertebral discs. Cell Tiss Res 289:185-190, 1997
- Sztralovics R, Alini M, Mort JS, Roughley PJ. Age-related changes in fibromodulin and lumican in human intervertebral discs. Spine 24(17):1765-71, 1999
- Bayliss MT, Johnstone B, O'Brien JP. Proteoglycan Synthesis in the Human Intervertebral Disc. Variation with Age, Region and Pathology. Spine 13(9): 972-981, 1988
- Aguiar DJ, Johnson SL Oegema Jr., TR. Notochordal Cells Interact with Nucleus Pulposus Cells: Regulation of Proteoglycan Synthesis. Exp Cell Res 246:129-137, 1999
- Oegema TR. Biochemistry of the intervertebral disc. Clin Sports Med. 12:419-39, 1993
- Aulisa L, Tamburrelli F, Lupparelli S, Tartarone M, Padua R. Immunohisto-chemical investigation on type III and VI collagen organization in human intervertebral discs in the neonatal period. Childs Nerv Syst 14(3):104-8, 1998
- Rufai A, Benjamin M, Ralphs Jr. The development of fibrocartilage in the rat intervertebral disc. Anat Embryol (Berl) 192(1):53-62, 1995

- Antoniou J, Goudsouzian NM, Heathfield TF, Winterbottom N, Steffen T, Poole AR, Aebi M, Alini M. The human lumbar endplate. Evidence of changes in biosynthesis and denaturation of the extracellular matrix with growth, maturation, aging, and degeneration. *Spine*. 21(10):1153-61, 1996
- 5 Thompson JP, Oegema TR Jr, Bradford DS. Stimulation of mature canine intervertebral disc by growth factors. *Spine*. 16:253-60, 1991
- Handa T, Ishihara H, Oshima H, Osada R, Tsuji H, Obata K. Effects of Hydrostatic Pressure on Matrix Synthesis and Matrix Metalloproteinase. Production in the Human Lumbar Intervertebral Disc. *Spine* 22(10):1085-1092, 1997
- 10 Ishihara H, McNally DS, Urban JPG, Hall AC. Effects of hydrostatic pressure on matrix synthesis in different regions of the intervertebral disk. *J Appl Physiol*. 80:839-846, 1996
- Miller JAA, Schmatz BS, Schultz AB. Lumbar Disc Degeneration: Correlation with Age, Sex and Spine Level in 600 Autopsy Specimens. *Spine*. 13(2):173-78, 1988
- Ozaki S, Muro T, Ito S, Mizushima M. Neovascularization of the outermost area of herniated lumbar 15 intervertebral discs. *J Orthop Sci*. 4(4):286-92, 1999
- Hadjipavlou AG, Simmons JW, Pope MH, Necessary JT, Goel VK. Pathomechanics and Clinical Relevance of Disc Degeneration and Annular Tear: a Point-of-View Review *Am J Orthop* 28:561-571, 1999
- Kawaguchi Y, Osada R, Kanamori M, Ishihara H, Ohmori K, Matsui H, Kimura T. Association Between 20 an Aggrecan Gene Polymorphism and Lumbar Disc Degeneration. *Spine* 24(23):2456-2460, 1999
- Kraemer J. Natural Course and Prognosis of Intervertebral Disc Diseases. *Spine* 20(6): 635-639, 1994
- Borenstein DG. Epidemiology, etiology, diagnostic evaluation, and treatment of low back pain. *Curr. 25 Opin. Rheumatol* 11:151-157, 1999
- Javedan SP, Dickman CA. Cause of adjacent-segment disease after spinal fusion. *Lancet* 354(9178):530-531, 1999
- Leivseth G, Salvesen R, Hemminghytt S, Brinckmann P, Frobin W. Do human lumbar discs reconstitute after chemonucleolysis? A 7-year follow-up study. *Spine* 24(4):342-7, 1999
- 30 Hou T-S, Tu K-Y, Xu Y-K, Li Z-B, Cai A-H, Wang H-C. Lumbar Intervertebral Disc Prosthesis. An Experimental Study. *Chin Med J* 104(5):381-386, 1991
- Enker P, Steffee A, Mcmillin C, Keppler L, Biscup R, Miller S. Artificial Disc Replacement. Preliminary Report with a 3-Year Minimum Follow-up. *Spine* 18(8): 1061-1070, 1993
- Bao Q-B, McCullen GM, Higham PA, Dumbleton JH, Yuan HA. The artifical disc: theory, design and 35 materials. *Biomaterials* 17:1157-1167, 1996
- Kostiuk, J.P. Intervertebral Disc Replacement, Experimental Study. *Clin. Orthop. Rel. Res.* 337: 27-41, 1997
- Zeegers WS, Bohnen LMLJ, Laaper M, Verhaegen MJA. Artificial disc replacement with the modular type SB Charite III: 2-year results in 50 prospectively studied patients. *Eur Spine J.* 8:210-17, 40 1999

- Choy DS. Percutaneous laser disc compression: 12 years experience with 752 procedure in 518 patients. *J Clin Laser Med Surg.* 16(6):325-31, 1998
- Nishida K, Kang JD, Suh J-K, Robbins PD, Evans CH, Gilbertson Lars G. Adenovirus-Mediated Gene Transfer to Nucleus Pulposus Cells *Spine* 23(22):2437-2443, 1998
- 5 Nishida K, Kang JD, Gilbertson LG, Moon S-H, Suh J-K, Vogt MT, Robbins PD, Evans CH. Modulation of the Biologic Activity of the Rabbit Intervertebral Disc by Gene Therapy: An In Vivo Study of Adenovirus-Mediated transfer of the Human transforming Growth Factor β 1 Encoding Gene *Spine* 24(23) 2419-2425, 1999
- Evans CH, Robbins PD. Genetically augmented tissue engineering of the musculoskeletal system. *Clin Orthop* 367(Suppl):S410-8, 1999
- 10 Ichimura K, Tsuji H, Matsui H, Makiyama N. Cell Culture of the Intervertebral Disc of Rats: Factors Influencing Culture, Proteoglycan, Collagen and Deoxyribonucleic Acid Synthesis. *J Spinal Disord* 4(4):428-436, 1991
- Maldonado BA, Oegema TR. Initial Characterization of the Metabolism of Intervertebral Disc Cells Encapsulated in Microspheres. *J Orthop Res* 10:677-690, 1992
- 15 Chelberg M, Banks GM, Geiger DF, Oegema TR. Identification of heterogeneous cell populations in normal human intervertebral disc. *J Anat* 186:43-53, 1995
- Chiba K, Andersson GB, Masuda K, Thonar E J-M A. Metabolism of the Extracellular Matrix Formed by Intervertebral Disc Cells Cultured in Alginate. *Spine* 22(24):2885-2893, 1997
- 20 Kusior LJ, Vacanti CA, Bayley JC, Bonassar LJ. Tissue Engineering of Nucleus Pulposis in Nude Mice. *Trans Orthop Society* 45:807, 1999
- Melrose J, Ghosh P, Taylor TKF. Proteoglycan heterogeneity in the normal adult ovine intervertebral disc. *Matrix Biology* 14: 61-75, 1994
- Inkinen RI, et al. Relative increase of biglycan and decorin and altered chondroitin sulfate epitopes in the degenerating human intervertebral disc. *J. Rheumatol* 25:506-514, 1998
- 25 Jahnke MR, McDevitt CA. Proteoglycans of the human intervertebral disc: Electrophoretic heterogeneity of the aggregating proteoglycans of the nucleus pulposus. *Biochem J* 251: 347-356, 1988
- Trout JJ, et al: Ultrastructure of the human intervertebral disc: II. Cells of the nucleus pulposus. *Anat Record* 204: 307-314, 1982
- 30 Sato M et al: Phenotype stability and loss of heterogeneity of rabbit intervertebral disc cells in three-dimensional culture. *Trans Orthop Res Soc* 24: 1026, 1999.
- Osada R et al: Autocrine/paracrine mechanism of insulin-like growth factor-1 secretion, and the effect of insulin-like growth factor-1 on proteoglycan synthesis in bovine intervertebral discs. *J Orthop Res* 14: 690-699, 1996
- 35 Kupchik HZ et al : A new method for the three-dimensional in vitro growth of human cancer cells. *Exp Cell Res* 147: 454-460, 1983
- Urban JPG, Maroudas A : Swelling of the intervertebral disc in vitro. *Connect Tissue Res* 9:1-10, 1981
- Oegema TR et al: Aggregated proteoglycan synthesis in organ cultures of human nucleus pulposus. *J Biol Chem* 254: 10579-10581, 1979
- 40 Boyle et al, *Osteoarthritis and Cartilage* 3, 117-125, 1995

WE CLAIM:

1. An engineered biological material comprising one or more tissues of intervertebral disc.
2. An engineered biological material as claimed in claim 1 characterized by a continuous layer of nucleus pulposus tissue.
- 5 3. An engineered biological material as claimed in claim 1 characterized by a continuous layer of annulus fibrosus tissue.
4. An engineered biological material as claimed in claim 1, 2, or 3 further characterized by the presence of extracellular matrix, large proteoglycans and collagen.
- 10 5. An engineered biological material as claimed in claim 2 or 4 wherein the continuous layer of nucleus pulposus tissue is on a substrate.
6. An engineered biological material as claimed in claim 3 or 4 wherein the continuous layer of annulus fibrosus tissue is on a substrate.
- 15 7. An engineered biological material as claimed in claim 5 or 6 wherein the substrate is selected from the group consisting of bone, an engineered biomaterial, preferably an engineered bone substitute, and porous tissue culture inserts.
8. Nucleus pulposus tissue obtained from an engineered biological material of claim 2 or 5.
9. Annulus fibrosus tissue obtained from an engineered biological material of claim 3 or 6.
10. An intervertebral disc construct comprising nucleus pulposus tissue as claimed in claim 8 or annulus fibrosus tissue as claimed in claim 9 fused to a substrate.
- 20 11. An intervertebral disc construct comprising nucleus pulposus tissue as claimed in claim 8 fused to a continuous layer of cartilage tissue on a substrate.
12. An intervertebral disc construct as claimed in claim 11 wherein the nucleus pulposus tissue is surrounded by a continuous layer of annulus fibrosus tissue.
- 25 13. A process for producing an engineered biological material as claimed in claim 1 comprising:
 - (a) isolating nucleus pulposus cells or annulus fibrosus cells from intervertebral disc;
 - (b) forming a layer of the nucleus pulposus cells or annulus fibrosus cells on a substrate, and;
 - (c) culturing the nucleus pulposus cells or annulus fibrosus cells in growth media under suitable conditions so that the nucleus pulposus cells or annulus fibrosus cells accumulate extracellular matrix and form a continuous layer of nucleus pulposus tissue or annulus fibrosus tissue.
- 30 14. A process for producing an engineered biological material as claimed in claim 1 comprising:
 - (a) isolating nucleus pulposus cells and annulus fibrosus cells from intervertebral disc;
 - (b) forming a layer of the nucleus pulposus cells surrounded by the annulus fibrosus cells on a substrate; and
 - (c) culturing the nucleus pulposus cells and annulus fibrosus cells in growth media under suitable conditions so that the nucleus pulposus cells and annulus fibrosus cells accumulate extracellular matrix and form nucleus pulposus tissue surrounded by annulus fibrosus tissue.
- 35

15. A process as claimed in claim 13 or 14 wherein the substrate is selected from the group consisting of bone, an engineered biomaterial preferably an engineered bone substitute, and porous tissue culture inserts.
16. An engineered biological material or intervertebral disc construct as claimed in any preceding claim 5 wherein cells of the nucleus pulposus tissue or annulus fibrosus tissue are transformed with recombinant vectors containing an exogenous gene encoding a biologically active protein that corrects or compensates for a genetic deficiency, or stimulates cell growth or stimulates extracellular matrix production by cells, or encodes a drug.
17. A system for testing a substance that affects nucleus pulposus cells or annulus fibrosus cells 10 comprising:
 - (a) generating or culturing an engineered biological material, or construct as claimed in any preceding claim in the presence of a substance which is suspected of affecting nucleus pulposus tissue or annulus fibrosus tissue; and
 - (b) determining the biochemical composition and/or physiological organization of tissue 15 generated or cultured with the biochemical composition and/or physiological organization of the biological material or construct generated or cultured in the absence of the substance.
18. A method of using an engineered biological material or construct as claimed in any preceding claim to test pharmaceutical preparations for efficacy in the treatment of diseases of intervertebral disc.
19. A method of replacing or repairing damaged or deficient intervertebral discs or portions thereof of a 20 patient comprising implanting an engineered biological material, nucleus pulposus tissue, annulus fibrosus tissue, or construct as claimed in any preceding claim into the site of the damaged or deficient intervertebral disc or portions thereof, of the patient.
20. A method for enhancing healing of an intervertebral disc in a patient which comprises inserting an engineered biological material, nucleus pulposus tissue, annulus fibrosus tissue, or construct as 25 claimed in any preceding claim into the site of a damaged intervertebral disc.
21. A method for repairing damaged or degenerated intervertebral discs comprising evacuating tissue from a nucleus pulposus portion of a degenerated intervertebral disc space, preparing an engineered biological material in accordance with a process as claimed in claim 13 using nucleus pulposus cells from the evacuated tissue, and implanting the engineered biological material in the space where the 30 tissue was evacuated.
22. A method for investigating the metabolism or degeneration of nucleus pulposus or annulus fibrosus cells or tissue using an engineered biological material or construct as claimed in any preceding claim as an *in vitro* model.
23. A gene therapy method comprising using an engineered biological material or construct as claimed in 35 any preceding claim.
24. Use of an engineered biological material, nucleus pulposus tissue, annulus fibrosus tissue, or construct as claimed in any preceding claim as an implant to replace or repair damaged or deficient intervertebral disc.

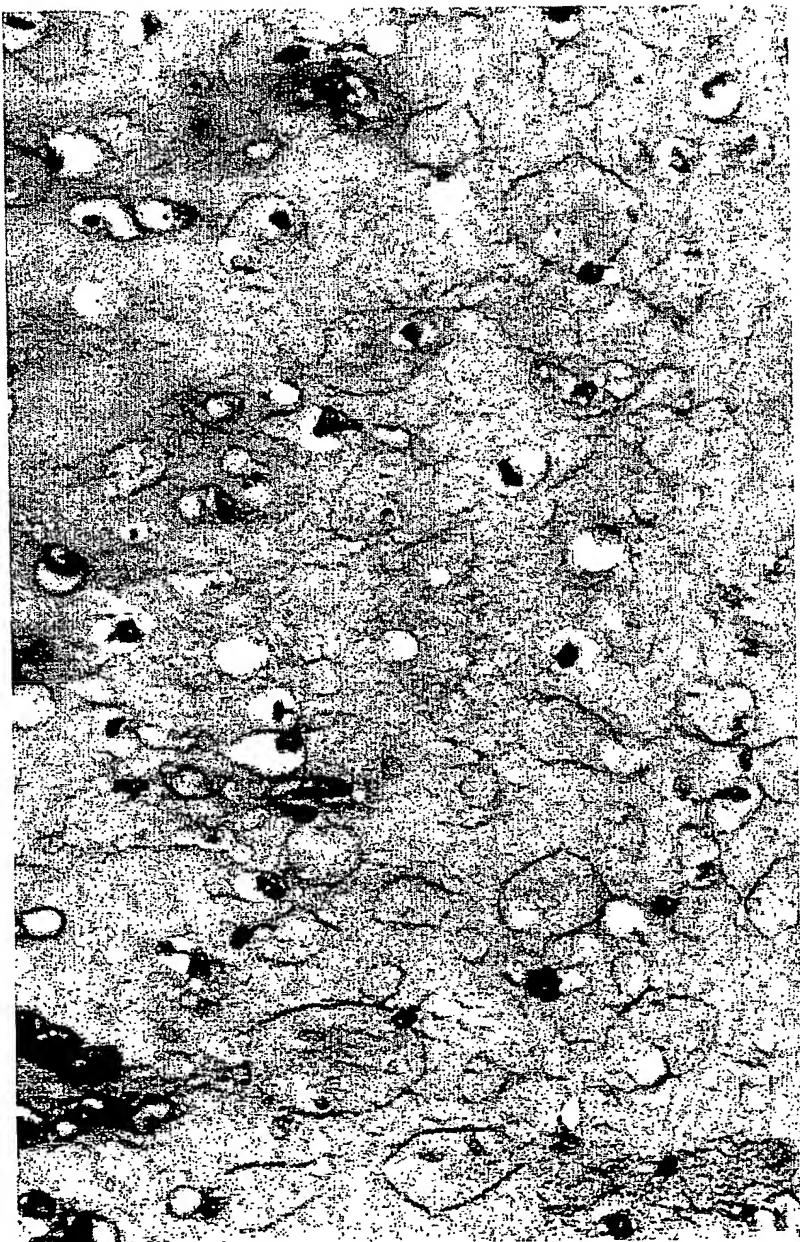
1/8

Figure 1A

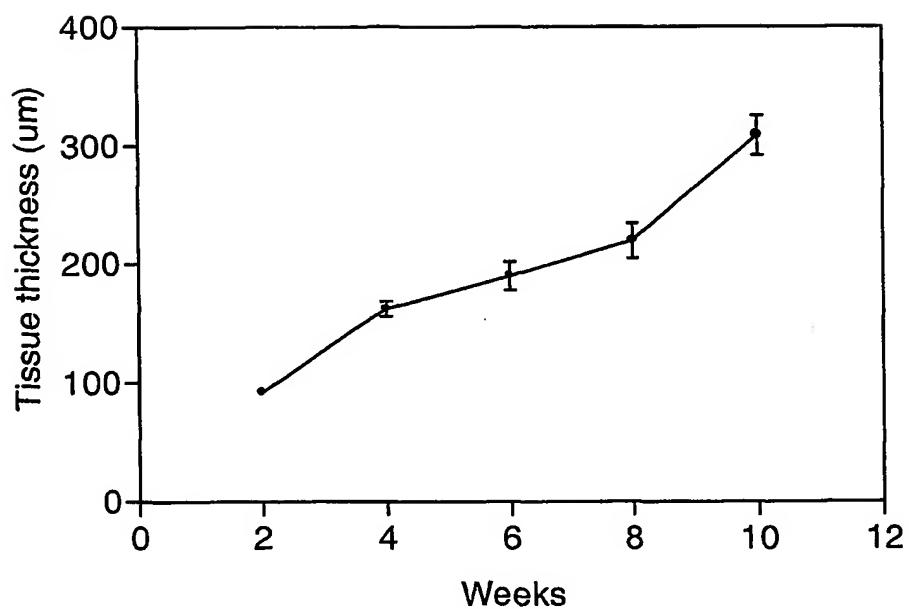


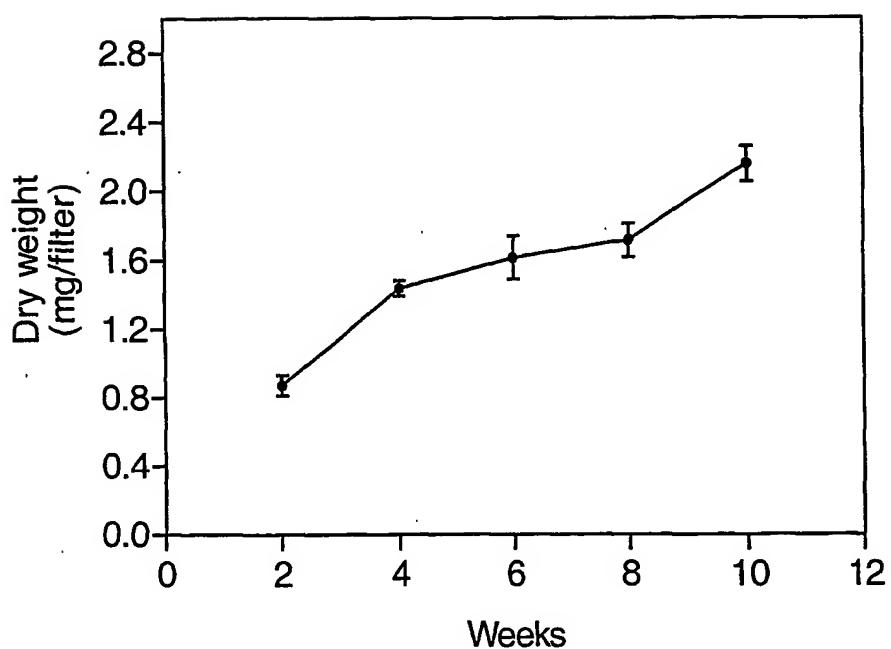
2/8

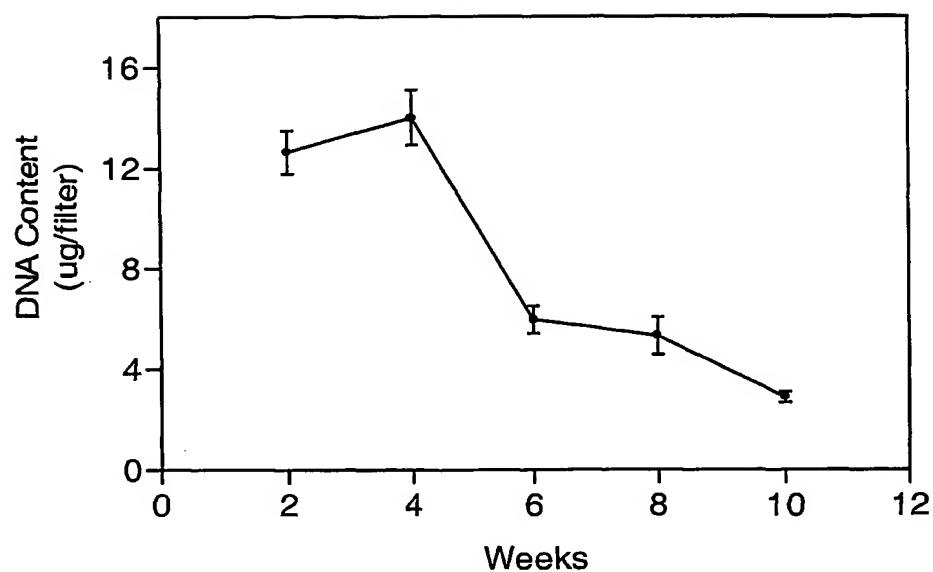
Figure 1B



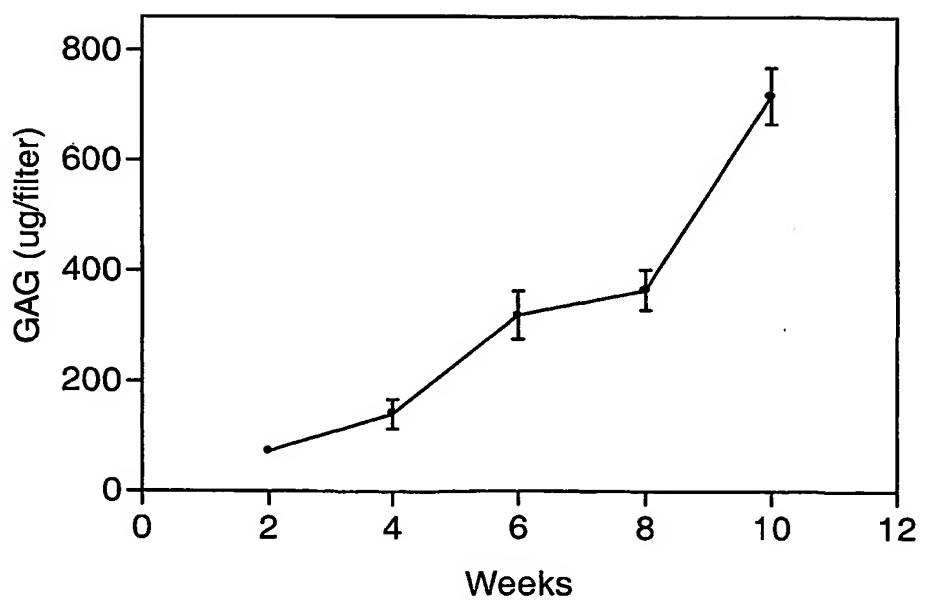
3/8

Figure 2

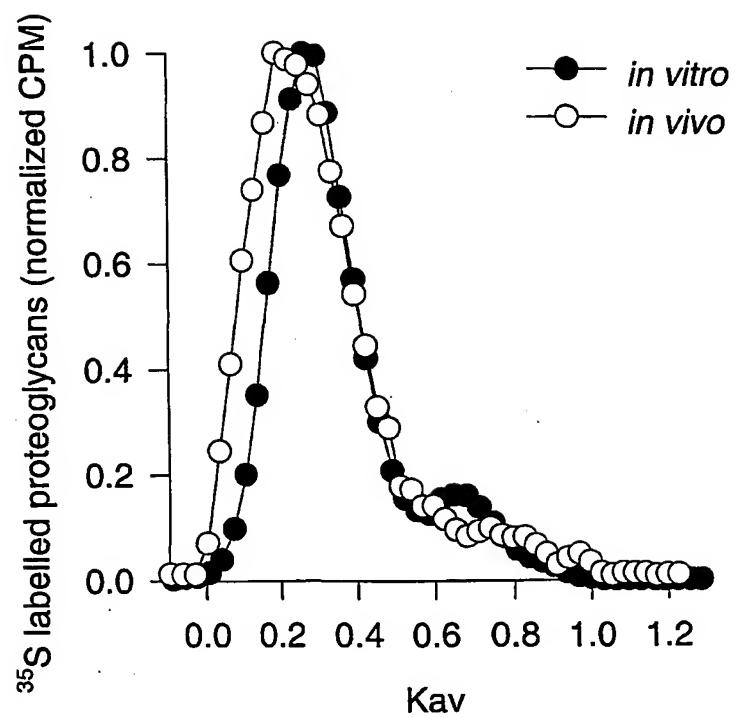
4/8**Figure 3**

5/8**Figure 4**

6/8

Figure 5

7/8

Figure 6

8/8

Figure 7